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The effect of historical legacy on adaptation: do closely related species respond to the environment in the same way?

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1 The effect of historical legacy on adaptation: do closely related species respond to the
2 environment in the same way?

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Abstract

The many documented examples of parallel and convergent evolution in similar environments are strong evidence for the role of natural selection in the evolution of trait variation. However, species may respond to selection in different ways; idiosyncrasies of their evolutionary history may affect how different species respond to the same selective pressure. To determine whether evolutionary history affects trait-environment associations in a recently diverged lineage, we investigated within-species trait-environment associations in the white proteas, a closely related monophyletic group. We first used MANOVAs to determine the relative importance of shared response to selection, evolutionary history, and unique responses to selection on trait variation. We found that on average, similar associations to the environment across species explained trait variation, but that the species had different mean trait values. We also detected species-specific associations of traits to the environmental gradients. To identify the traits associated uniquely to the environment we used a structural equation model. Our analysis showed that the species differed in how their traits were associated with each of the environmental variables. Further, in the cases of two root traits (root mass and root length:mass ratio), two species differed in the direction of their associations (e.g. populations in one species had heavier roots in warmer areas, and populations in the other species had lighter roots in warmer areas). Our study shows that even in a closely related group of species, evolutionary history may have an effect on both the size and direction of adaptations to the environment.

Keywords: *Protea*, evolutionary history, adaptation, structural equation model

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Introduction

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The many examples of convergence and parallel evolution in similar environments are some of the most striking examples of evolution by natural selection, suggesting that species respond similarly to the same environmental selection pressures. The repeated evolution of succulence in plants from arid environments, for example, reflects the consequences of natural selection for water storage in arid environments (Beard 1976, Ogburn & Edwards 2010). Similarly, the repeated evolution of benthic and limnetic forms of fish (sticklebacks, lake whitefish, pumpkinseed, and bluegill sunfish) in post-glacial lakes is the result of character displacement in response to competition in the newly created lake habitats (reviewed by Schluter & McPhail 1993).

We recognize less often that species may respond to the same selection pressure in different ways. For example, within *Drosophila melanogaster*, Cohan and Hoffman (1986) found similar responses to selection for ethanol tolerance in replicate experimental populations, but the genetic underpinnings of the ethanol tolerance differed from one replicate to another. The fitness associated with different genotypes at the alcohol dehydrogenase locus depended on the pre-existing genetic background of the individuals. Similarly, Young *et al.* (2007) showed that shrews with similar diets have functionally similar, but morphologically divergent, jaw structures. Thus, different morphologies may also be the result of similar selection (Young *et al.* 2007). Even in cases where convergent evolution is well documented, the unique evolutionary history of individual lineages may play a role in how each lineage adapts to its environment. For example, in the radiation of *Anolis* lizards on Caribbean islands, crown giant anoles on the island of Puerto Rico tend to have narrower pectoral width, deeper heads, and wider pelvic widths

60 than crown giant anoles on other islands (Langerhans *et al.* 2006). There are even
61 examples where apparently adaptive responses to the same environmental selection arise
62 from trait responses in opposite directions. For example, Nakazato *et al.* (2008) found
63 that in two species of Andean tomatoes, the between-population associations of plant
64 height with elevation and of days to wilting with mean annual precipitation were similar
65 between species. In contrast, the association of leaf area with elevation depended on the
66 species examined. In *Solanum pimpinellifolium*, plants tended to have larger leaves in the
67 higher elevation population, and in *Solanum lycopersicum* var. *cerasiforme* plants tended
68 to have smaller leaves in the higher elevation population. Differential responses to
69 apparently similar selection pressures could reflect lineage-specific differences in genetic
70 variances and covariances between traits (Arnold *et al.* 2008, Hanson & Houle 2008), the
71 vagaries of genetic drift and independent mutational histories (Travisiano *et al.* 1995), or
72 subtle differences in the environment that were not measured. Different adaptive
73 responses, although affected by non-adaptive forces, are ultimately driven by natural
74 selection to a landscape that has multiple optima.

75 Large-scale trait-environment associations are often attributed to the effects of
76 natural selection. For example, plants from drier and more nutrient poor areas tend to
77 have thicker or denser leaves (Niinemets 2001, Cunningham *et al.* 1999, Fonseca *et al.*
78 2000), plant height is correlated with precipitation (Moles 2009), and rooting depth
79 increases with increasing drought (Canadell *et al.* 1996) and rainfall seasonality (Schenk
80 & Jackson 2002). All of these associations have been interpreted as adaptive responses to
81 the environment, and these large-scale patterns suggest that species respond predictably
82 to variation along these environmental gradients. Similarly, trait-environment

83 associations among populations within species are often interpreted as adaptations to the
84 local environment (Endler 1986), but the extent to which these large scale patterns are
85 reflected within species is less explored.

86 In this study we investigated a small evolutionary radiation to determine whether
87 trait variation among populations within species is associated with similar environmental
88 gradients across species and whether this trait variation reflects large-scale trait-
89 environment associations (Table 1). Specifically, we investigated trait-environment
90 associations within the white proteas (*Protea* sect. *Exsertae*), a group of six closely
91 related species endemic to the mountains of South Africa that diverged recently (0.34-1.2
92 million years; Valente *et al.* 2010). While the species are almost completely allopatric,
93 they have broad environmental tolerances (Latimer *et al.* 2009), and they do not cleanly
94 partition environmental space (Figure 1; see also Latimer 2006). In fact, as Latimer *et al.*
95 (2009) showed, the differences between species in their mean environments is not enough
96 to account for the almost complete allopatry in this group, suggesting that adaptive
97 differences among populations --in addition to differences among species-- may
98 contribute significantly to the diversity of the group (Carlson *et al.* 2011).

99 Our previous work on the white proteas found that among-population differences
100 in leaf traits are adaptively associated with climate, e.g., thick or dense leaves appear to
101 be adaptations to a harsh climate (Carlson *et al.* 2011). These results are consistent with
102 expectations from large-scale surveys of trait-environment correlations across all
103 flowering plant species (Wright *et al.* 2004). Differences in traits associated with access
104 to nutrients and water and differences in seed size are also likely important foci of
105 adaptation in most plants, including the white proteas. The environments in which the

106 white proteas grow vary widely in the timing and amount of rainfall (Schulze 2007), in
107 hydrological conditions, and in soil nutrients (Specht & Moll 1983; Witkowski &
108 Mitchell 1987). In proteas, long tap roots are needed to reach the water table (Richards *et*
109 *al.* 1995, Watt & Evans 1999), and access to nutrients is facilitated by the production of
110 proteoid roots; specialized cluster roots on lateral roots. Proteoid roots are especially
111 important for uptake of phosphorous (Lamont 1982, Neumann & Martinoia 2002), but
112 they also enhance uptake of nitrogen and micronutrients (Jeschke & Pate 1995).
113 Similarly, large seeds have been found to be adaptive in low nutrient conditions (due to
114 better provisioning; Beadle 1966), both within (Bonfil & Kafkafi 2000, Vaughton &
115 Ramsey 2001) and across species (Milberg *et al.* 1998, Shane *et al.* 2008).

116 Here we use leaf, shoot, and root traits measured in a common environment to
117 answer the following questions: 1. Does evolutionary history, similar evolutionary
118 responses to the environment, or lineage-specific evolutionary responses to the
119 environment play a larger role in the trait diversification of the white proteas? 2. What
120 traits are associated with lineage-specific evolutionary responses to the environment? 3.
121 Do the species-specific trait-environment associations reflect large scale trait-
122 environment correlations?

123

124 **Materials and Methods**

125 *Study Species*

126 The white proteas are a well-supported, monophyletic clade within the genus *Protea*
127 (*Protea* section *Exertae*, Valente *et al.* 2010). The current taxonomic treatment
128 recognizes 6 species, *P. aurea* (Burm. f.) Rourke ssp. *aurea* (shuttlecock sugarbush), *P.*

129 *aurea* ssp. *potbergensis* (Rourke) Rourke (potberg sugarbush), *P. lacticolor* Salisb.
130 (hottentot sugarbush), *P. mundii* Klotzsch (forest sugarbush), *P. punctata* Meisn. (water
131 sugarbush), *P. subvestita* N.E. Br. (waterlily sugarbush), and *P. venusta* Compton
132 (creeping beauty). The clade is endemic to Southern Africa, and all of the species but *P.*
133 *subvestita* are endemic to the Cape Floristic Region (CFR) of southwestern South Africa.
134 *P. subvestita* is native to the Eastern Cape and Kwa-Zulu Natal provinces, as well as
135 Lesotho (Figure 2). The species of the white proteas radiated quickly after the origin of
136 the group, and phylogenetic distance between populations and species is small (Prunier &
137 Holsinger 2010).

138 All of the white proteas are sclerophyllous evergreen shrubs. *P. venusta* has a
139 sprawling habit, but the remaining species are all ecologically similar. They grow upright
140 and up to 4 meters tall. They are killed by fire, regenerating from seeds stored in
141 serotinous cones (Rebelo 2001). They have largely allopatric ranges, although some
142 populations of *P. punctata* and *P. venusta* are found in close proximity at the tops of the
143 Swartberg and Kammannassie mountains. All are presumed to be pollinated by
144 sugarbirds and sunbirds (Rebelo *et al.* 1984, Rebelo 2006, de Swardt 2008, Carlson &
145 Holsinger 2010). Because their flowering periods overlap and most species share bird
146 pollinators, the white proteas often hybridize when grown together in cultivation and
147 when they co-occur in the wild (Rourke 1980, Prunier & Holsinger 2010).

148 *Sampling Protocol*

149 We collected seeds from wild populations in February – April 2008. We sampled 5-6
150 populations from each species (total populations=30), spanning most of the geographic
151 range of each species (Figure 2, Supplemental Table 1). We excluded *P. aurea* ssp.

152 *potbergensis* from this study because we were able to sample only two populations of this
153 geographically restricted sub-species. Within each population, we collected 5-8
154 seedheads from 8 plants approximately 10 meters apart along a linear transect through the
155 population. We dried the seedheads in a cold room maintained at low humidity until they
156 opened and the single-seeded fruits could be removed. We then selected fruits containing
157 potentially viable seeds by identifying fruits in which the seed was filled and free of
158 insect damage. We stored the fruits containing potentially viable seeds (henceforth seeds)
159 at room temperature until planting.

160 *Greenhouse Experiment*

161 We weighed, surface sterilized with 10% bleach, and planted one seed from eight
162 maternal lines per population into each of six standard 288 plug flats (TLC Plastics or
163 similar) filled with a standard nursery soil mix in a complete random block design in
164 September 2008. We moved the seeds to a growth chamber (Conviron, Winnipeg
165 Canada) programmed for short warm days and cool long nights (10 hours: 20 °C, 14
166 hours: 8 °C) to simulate autumn in the southern hemisphere (April-June), when the seeds
167 germinate in the wild (Rebelo 2001) and assessed germination every day until 47 days
168 after planting, at which point germination had slowed to less than one plant every other
169 day. One or two days after germination, we moved 2 seedlings from each maternal line to
170 pots 10 cm × 10 cm wide at the top and 76.2 cm tall (Stuewe and Sons Inc., Tangent OR)
171 in a greenhouse in Storrs, CT. The pots contained a soil mix with 5 parts peat : 4 parts
172 sand : 2 parts fine perlite : 1 part charcoal. This soil mix is modified from a standard low
173 nutrient greenhouse mix by the addition of sand to provide better drainage. We randomly
174 assigned seedlings to pots within the greenhouse. Supplemental light (80-150 $\mu\text{mol}/\text{m}^2\text{s}$)

175 was added 30 minutes after sunrise and ended 30 minutes before sunset to ensure an even
176 light environment across the greenhouse while maintaining natural red/far red ratios at
177 dusk and dawn. This experiment was performed in the autumn and early winter in the
178 northern hemisphere (September to December) at a latitude higher than the plants' native
179 ranges (41° N vs. 33° S). As a result, the days were shorter than they would experience in
180 the wild, but by less than an hour per day through the duration of the experiment.

181 We kept the pots moist for the first month after transplanting; after that, we
182 watered pots with tap water when they became dry. Due to uncertainty in optimal
183 growing conditions, some plants (4 per population) received no fertilizer. All other plants
184 received 100ml of no-phosphorous fertilizer (60ppm 20-0-20, J. R. Peters, Inc.,
185 Allentown, PA) once every other week. Plants in the no fertilizer treatment received
186 100ml of tap water instead.

187 *Harvesting and Excavation*

188 All of the plants were excavated in December 2008, 77-83 days after planting. Before the
189 soil was disturbed, we removed the shoot at the soil surface, measured its height and
190 removed a leaf for area and dry weight measurements. We measured fresh leaf area using
191 a LiCor 3100 leaf area meter (LiCor, Lincoln NE) and dry leaf and shoot (stem and
192 leaves) mass after drying leaves and shoots in an oven for two weeks at 60 °C. We
193 extracted the roots by slicing the pots lengthwise and gently teasing the roots from the
194 soil. Some fine roots were lost in extraction. Once the entire root was extracted from the
195 pot, we washed off the remaining soil, measured the length of the longest root and noted
196 the presence or absence of root rot and proteoid roots. Finally, we measured dry weight
197 of the roots after drying them for two weeks at 60 °C. From these primary measurements

198 we calculated several derived traits: specific leaf area (SLA, leaf area/ leaf dry mass), leaf
199 length:width ratio (LWR, leaf length/leaf width), root:shoot biomass ratio (Rt:ShtM, root
200 mass/shoot mass), root:shoot length ratio (Rt:ShtL, root length/shoot height), and root
201 length:root mass (RtL:M, length of longest root/root mass). The leaf traits and the
202 root:shoot ratios are widely used functional traits, and root length:mass ratio captures
203 important variation in root allocation strategies. Plants that died or had substantial root rot
204 were excluded from the analysis (<8% of plants), resulting in a total of 384 plants (91 no
205 fertilizer, 293 fertilizer).

206 *Climate and Soil Data*

207 To extract climate variables for populations used in the study, we intersected the
208 locations of the sampled populations with layers from the South African Atlas of
209 Agrohydrology, based on more than thirty years of weather data from more than 1000
210 weather stations (Schulze 2007). We reduced the large number of available climate
211 variables to a manageable number by choosing broad environmental categories that we
212 expected to differ among species based on Latimer *et al.*'s (2009) work in the white
213 proteas. We then performed separate principal components analyses on variables
214 corresponding to these categories. This resulted in one unmanipulated and two composite
215 variables that encapsulate variation in the categories that have been found to be important
216 in delineating white protea distributions (Latimer *et al.* 2009): seasonal rainfall
217 concentration (PPTCON), winter temperature (COLDPCA), and intensity of dry season
218 drought (DRYPCA). PPTCON ranges from 0 (even rainfall) to 100 (all of the yearly
219 rainfall falling in one month). PPTCON is positively correlated with total rainfall, which
220 we did not include in our analysis ($r = 0.51$). COLDPCA is a measure of the coldness of

221 winters and is strongly negatively correlated with elevation ($r = -0.61$). This axis was
222 calculated as the first axis of a PCA of the average minimum daily temperature in the
223 coldest month and the number of heat units in the coldest three months (the first axis
224 explained 87% of variation in these two variables). Large values indicate warmer winters.
225 DRYPCA is the first axis of a PCA of the number of days with less than 2mm rainfall in
226 the driest three months and the total rainfall during those months (87% of variation in
227 these two variables). Large values indicate milder, moister dry seasons. These
228 environmental variables are the same as those used in Carlson *et al.* (2011).

229 To gauge the degree to which plant traits are associated with the soil fertility of
230 their home environments, we collected bulked soil samples at 15 and 30 cm depth from 3
231 locations at each seed collection site. Bulk samples were dried for 1 week at 60 °C and
232 analyzed at BEM Labs (Somerset West, SA) for percent N, total P, total K, pH and cation
233 exchange capacity. We constructed a final PCA, FERTPCA, from these NPK
234 concentrations (variable loadings: percent N 0.659, total P 0.496, total K 0.588, first axis
235 explains 58.7% of variation). Values for the environmental variables for each population
236 can be found in Supplemental Table 1.

237 *1. Does evolutionary history, similar responses to the environment, or unique responses*
238 *to the environment play a larger role in the trait diversification of the white proteas?*

239 To determine the relative contributions of evolutionary history, shared responses
240 to the environment, and unique responses to the environment to trait diversity, we
241 conducted MANOVAs in R (manova, R Core Development Team 2011). The MANOVA
242 approach allows us to account for correlations among the response variables (all plant
243 traits listed above) while testing their relationships with environmental gradients, the

244 species effect, and the interaction between species and environment. We standardized the
245 environmental predictors so that each one had a mean of zero and a standard deviation of
246 1. This allows us to investigate the relative importance of evolutionary history (species
247 effect), shared response to the environment (environment effects: FERTPCA,
248 COLDPCA, DRYPCA and PPTCON), and unique response to the environment (all
249 species \times environment interactions). For this analysis, we included only the individuals
250 which received fertilizer in the greenhouse, for a total of 293 individuals.

251 We estimated the relative importance of each factor by calculating the partial
252 Wilks's η^2 for each factor. Wilks's η^2 is a measure of the partial variance explained by a
253 factor and the multivariate approximation of $SS_{\text{effect}}/(SS_{\text{effect}} + SS_{\text{error}})$; (see
254 Langerhans & DeWitt 2004 for further discussion of partial η^2). To create confidence
255 intervals around the estimates of η^2 , we jackknifed the analysis by sequentially dropping
256 one individual from the analysis (Davison & Hinkley 1997). We estimated the marginal
257 SS for each factor as the SS of the last factor added to the model, rotating the order of
258 factors included so that each appeared in the final term. We used this marginal SS to
259 estimate η^2 . Because interaction terms are always estimated after main effects, we
260 removed the interaction terms from the model to estimate the η^2 of each of the main
261 factors. The main factor η^2 s reflect the variance in multivariate trait space that is due to
262 the average response of species to the four environmental variables and the variance that
263 is explained by the average trait differences between species.

264 *2. Which traits drive any unique response to the environment?*

265 We are interested in the relationships of many traits to the environment. However,
266 these traits did not evolve independently of one another and developmental responses

267 within an individual are likely to be correlated across several traits. Separate multiple
268 regressions would allow us to investigate the association between individual traits and
269 environmental covariates (as in Nakazato *et al.* 2008, Carlson *et al.* 2011). However, they
270 would not allow us to differentiate between direct associations between a trait and the
271 environment and indirect associations that arise because a particular trait is
272 developmentally or genetically correlated with another trait that has a direct association.
273 We use a structural equation model of trait variation to account for trait-trait associations,
274 allowing us to isolate direct associations of traits with environmental features. Structural
275 equation modeling (Jöreskog 1970; Jöreskog & Sörbom 1996; Lee 2007) provides a
276 general approach to statistical analysis of unobservable, “latent”, variables by specifying
277 their relationship to observable, “manifest”, variables. By including these latent variables,
278 our model accounts for trait-trait associations and allows us to estimate only 28
279 relationships (lines in Figure 3) rather than trying to examine all 105 pairwise
280 relationships among 15 traits.

281 We analyze the coordinated response of whole plants in terms of four
282 components: root, shoot, leaf, and root:shoot. We are especially interested in these four
283 components because together they represent the three fundamental components of the
284 plant body (root, shoot, and leaf) and root:shoot allocation has long been recognized as a
285 fundamental axis associated with plant adaptation (Orians & Solbrig 1977, Chapin 1980,
286 Lloret *et al.* 1999). These are the four latent variables (LV) identified in the center of
287 Figure 3. Each of the manifest variables is related to one of the latent variables and to
288 environmental covariates through a linear regression. For example, if $rootlg_i$ is the root
289 length of the i th individual in the sample, then

290 $rootlg_i = \alpha_{species[i]} + \beta_{rootlg.root} root_i + \mathbf{g}_{species[i]} \mathbf{env}_{pop[i]} + \varepsilon_{bin[i]} + \varepsilon_i$,

291 where a is the species-specific intercept; $\beta_{rootlg.root}$ is the “loading” of $rootlg$ on the root
292 latent variable, $\mathbf{g}_{species[i]}$ is the species-specific vector of regression coefficients on the
293 environment associated with individual i , $\mathbf{env}_{pop[i]}$ is the vector of environmental
294 covariates associated with the site of origin for individual i , $\varepsilon_{bin[i]}$ is the random effect
295 associated with the greenhouse bin in which individual i was grown, and ε_i is the random
296 error associated with individual i . Notice that the value of the root latent variable is
297 defined only implicitly through its set of regression relationships with corresponding
298 manifest variables, in the case of $root$ those regressions are the ones involving $root\ mass$,
299 $root\ length$, $proteoid\ roots$, $main\ root\ dead$, and $root\ length:mass\ ratio$. To ensure a
300 unique value for each latent variable, the loading of one manifest variable is set to 1, in
301 our case $root\ length$ (root LV), the $presence\ of\ leaves$ (shoot LV), SLA (leaf LV), and
302 $root:shoot\ weight$ (root mass/shoot mass, root:shoot LV). The choice of variable used for
303 standardizing the latent variable relationships is arbitrary, because remaining loadings are
304 estimated relative to the loading of 1 for the variable used for standardization. The
305 loadings represent the extent to which each of the manifest variables is associated with
306 the latent variable. Correlations between manifest variables are accounted for through
307 their association with the latent variables.

308 Our data include a mixture of continuous and binary response variables. We used
309 JAGS 2.1.0 (Plummer 2003) to analyze the structural equation model in a Bayesian
310 framework using a logistic link for binary response variables and diffuse normal priors on
311 all loadings and regression coefficients. As suggested by Gelfand *et al.* (1995), we use
312 hierarchical centering to improve convergence of the MCMC sampler. We report results

313 from an analysis using 4 independent chains, each with a burn-in of 50,000 iterations,
 314 followed by samples taken at 160 step intervals for the next 200,000 iterations, for a total
 315 of 5000 samples from the posterior distribution. Standard convergence diagnostics
 316 (Gelman & Rubin 1992) suggest that convergence was satisfactory. All Rhat values are
 317 less than 1.04, most were less than 1.005, and the effective size of the sample from the
 318 posterior distribution of each parameter ranged between 100-5000. For more than 90% of
 319 the parameters, the effective sample size was greater than 500.

320 The model output includes the full posterior distribution for all parameters, which
 321 we summarize with the posterior means and 95% credible intervals. Estimates for which
 322 the 95% credible interval does not overlap zero are statistically distinguishable from zero.
 323 As an additional test of the importance of unique responses to the environment we
 324 examined two models, one that imposes the same trait-environment association across all
 325 species (the common-trait model) and one that allows each species to exhibit a different
 326 trait-environment association (the varying-trait model). The common-trait model allows
 327 each species to have a different mean trait value, but forces the regression coefficients
 328 describing trait-environment relationships to be the same for all species. This model can
 329 be summarized as

$$330 \text{ Trait } Z_i = \alpha_{\text{species}[i]} + \beta_{\text{trait:latent}} \text{latent} + \mathbf{g} \bullet \mathbf{env}_{\text{pop}[i]} + \varepsilon_{\text{bin}[i]} + \varepsilon_i$$

331 where $\alpha_{\text{species}[i]}$ is a species-specific intercept and \mathbf{g} is vector of regression coefficients
 332 shared by all species. The varying-trait model can be summarized as:

$$333 \text{ Trait } Z_i = \alpha_{\text{species}[i]} + \beta_{\text{trait:latent}} \text{latent} + \mathbf{g}_{\text{species}[i]} \bullet \mathbf{env}_{\text{pop}[i]} + \varepsilon_{\text{bin}[i]} + \varepsilon_i$$

334 where $\mathbf{g}_{\text{species}[i]}$ is a vector of species-specific regression coefficients associated with the
 335 species to which individual i belongs.

336 We compared the adequacy of these models using DIC, an information criterion
337 similar to AIC (Spiegelhalter *et al.* 2002). AIC cannot be used for model choice in a
338 Bayesian context, because it depends on a maximum-likelihood estimate for the
339 parameters. DIC is the Bayesian equivalent, including a component measuring the fit of
340 the model to the data and a component describing the complexity of the model. A
341 difference in DIC larger than 10 units indicates that one model is strongly preferred over
342 the other. If the preferred model is the one in which the species are allowed to respond
343 differently to the environmental gradients, we have evidence that species have different
344 trait – environment relationships. We investigate those differences further by identifying
345 the relationships for which the credible interval does not overlap zero. This is a
346 conservative approach to detecting differences because the failure to detect an effect may
347 reflect a lack of statistical power rather than the absence of an effect. Furthermore,
348 because estimates for individual species trait-environment relationships emerge from a
349 hierarchical model in which species means are drawn from a common distribution, a
350 correction for multiple comparisons is not necessary (Gelman and Hill 2007). In order to
351 retain as large sample size as possible (N=384), all of the plants were included in this
352 analysis. The effect of the fertilizer was included in the model and does not effect the
353 results reported.

354 **Results**

355 *1. Does evolutionary history, similar responses to the environment, or unique responses*
356 *to the environment play a larger role in the trait diversification of the white proteas?*

357 The MANOVA revealed significant effects for all three classes of factors; shared
358 response to the environment, unique responses to the environment, and evolutionary

359 history (Table 2). Much of the morphological variance among populations was explained
360 by the model, as shown by the Wilks's η^2 values for the species and direct environmental
361 effects. Based on the estimates of partial variance explained (Wilks's η^2) and the
362 measures of significance, historical effects and shared response to the environment were
363 similarly important in predicting trait variation. The factor that explained the most
364 variance was shared response to rainfall seasonality, followed by the effect of history
365 (species effect). The unique responses to selection were significant, but mostly smaller
366 than the shared responses to selection. However, the effect of unique responses to winter
367 temperature ($\eta^2=0.140$) was nearly as large as those of shared responses to winter
368 temperature ($\eta^2=0.188$).

369 2. *Which traits drive the unique response to the environmental gradients?*

370 The trait-environment correlations are underpinned by the structure of the structural
371 equation model, which is detailed in Table 3 and Figure 3. All of the manifest variables
372 were significantly related to the latent variables. The fertilizer treatment had an effect
373 only on the root latent variable.

374 With a delta DIC of -218.3, the model that allows trait-environment relationships
375 to differ among species (DIC=12152.0) is much more strongly supported than the model
376 in which the species are forced to respond similarly to the environment (DIC=12370.3).
377 The significant species-specific trait-environment correlations show why this is the case
378 (Figure 4, Supplemental Table 2). We detected species-specific trait-environment
379 correlations in all species but *P. venusta*. We identified the most species-specific
380 relationships in *P. mundii*, with 20 out of the 51 significant trait-environment associations
381 occurring between *P. mundii* populations. Winter temperature (COLDPCA) was the

382 environmental axis most frequently involved in species-specific trait-environment
383 associations with 21 significant trait-environment associations between traits and
384 COLDPCA. Leaf traits varied the most with winter temperature (COLDPCA) and
385 summer drought (DRYPCA). Root traits varied the most with winter temperature
386 (COLDPCA) and rainfall concentration (PPTCON). Associations with shoot, root:shoot,
387 and seed traits were more evenly distributed across the environmental axes.

388 When all (both significant and non-significant) trait-environment associations are
389 considered, species only responded similarly in four of the 60 of the trait-environment
390 combinations (Supplemental Figure 1). Across all species, seeds were always heavier in
391 populations from wetter areas, leaves always had larger areas in wetter areas, shoots were
392 always taller in populations from areas with less seasonal rainfall, and leaves were wider
393 (lower LWR) in areas with more concentrated rainfall.

394 The dissimilarity between species in how their traits vary with their environment
395 becomes even more apparent when only the significant associations are considered. For
396 example, leaf traits in *P. aurea*, *P. laticolor*, *P. punctata*, and *P. subvestita* vary along a
397 gradient in the intensity of summer drought (DRYPCA), but the identity of those traits
398 differs among species. In *P. aurea* and *P. laticolor* leaf shape (LWR) varies across the
399 gradient of summer drought, while in *P. punctata* and *P. subvestita* leaf mass varies. In
400 other cases, the same trait varies across an environmental gradient in more than one
401 species, but the direction of the associations differ among species. For example, *P.*
402 *mundii* and *P. punctata* individuals from warmer areas tend to have heavier root systems,
403 but *P. aurea* individuals from warmer areas tend to have lighter root systems.

404 All of the cases in which species differed significantly in the direction of their
405 relationships between a trait and the environment were root traits (arrows in Figure 4). In
406 addition to the differences in root biomass described above, *P. mundii* tends to have more
407 branched root systems (low root length to mass ratio) in warmer areas, while *P. aurea*
408 tends to have less branched root systems (higher root length to mass ratio) in warm areas.
409 Also, *P. mundii* tends to have more branched roots in areas with more concentrated
410 rainfall, but *P. aurea* has less branched roots in areas with more concentrated rainfall.

411 In addition to the opposite responses to COLDPCA and PPTCON listed above,
412 other root traits were correlated with the environmental gradients. They were associated
413 with rainfall seasonality, but the pattern of association varied markedly among species. *P.*
414 *mundii* had the most varied root response to rainfall seasonality, with fewer proteoid
415 roots, heavier roots, and more branched roots in areas with more seasonal rainfall. *P.*
416 *subvestita* individuals from areas with more seasonal rainfall had shorter roots. Root traits
417 also varied in response to intensity of summer drought, but only in *P. subvestita*, in which
418 individuals from wetter areas tended to have heavier, more branched root systems.
419 Species also differed in the association between root traits and winter temperature. In
420 addition to the conflicting trends in *P. aurea* and *P. mundii* described above, *P. punctata*
421 had heavier root systems in warmer areas. We failed to detect significant associations
422 between any root traits and soil fertility.

423 Seed traits did not vary predictably along the environmental gradients we studied.
424 We expected to find heavier seeds in lower nutrient areas (Bonfil and Kafkafi 2000), but
425 we detected no relationships between seed mass and soil fertility (FERTPCA). While
426 seed mass did vary in association with winter temperature and rainfall, the nature of the

427 association differed among species. In *P. aurea*, populations from warmer areas had
428 smaller seeds. In *P. mundii*, populations from areas with less seasonal rainfall had smaller
429 seeds. *P. laticolor* and *P. mundii* populations from areas with warmer winters
430 germinated faster.

431 Similarly, root:shoot ratios rarely varied with the environmental gradients in the
432 direction we expected based on many studies of plant allocation strategies in other
433 systems (Orians and Solbrig 1977, Schenk and Jackson 2002). Root:shoot ratios are
434 expected to be higher when water availability is low (McCarthy & Enquist 2007), but we
435 detected such a relationship only in *P. mundii*. In areas with less intense droughts, *P.*
436 *mundii* individuals invested more biomass in shoots than in roots. In *P. aurea* and *P.*
437 *laticolor*, root:shoot ratio was associated with winter temperature, but not with summer
438 drought. Individuals in these species from areas with warmer winters tended to invest
439 more in shoots than roots.

440 Shoot traits were quite labile in response to the environmental gradients. *P.*
441 *mundii*, *P. laticolor*, and *P. punctata* individuals from warmer areas tended to be bigger
442 (more likely to have leaves, have heavier shoots, or taller shoots). *P. mundii* and *P.*
443 *subvestita* individuals from more fertile areas tended to be larger as well. However, *P.*
444 *mundii*, *P. aurea*, and *P. punctata* individuals from areas with more concentrated rainfall
445 tended to be smaller.

446 Many of the trait-environment relationships that we detected in leaves were as
447 expected. For example, we found wider leaves in wetter areas (Cunningham *et al.* 1999)
448 in *P. aurea* and *P. laticolor*, and heavier leaves in populations from wetter areas in *P.*
449 *mundii*, *P. punctata*, *P. subvestita*. The one environmental association that we found with

450 SLA, widely thought to be an important adaptation to many environmental stresses, was
451 also in the direction that we expected. We found that in *P. aurea*, plants from colder sites
452 tended to have lower SLA, thicker or more dense leaves. *P. mundii* and *P. punctata*
453 populations from colder areas also tended to have smaller leaves.

454 **Discussion**

455 Large scale trait-environment associations and the myriad examples of parallel
456 evolution and convergence show that species often respond similarly to similar selection
457 pressures. However, the vagaries of evolutionary history also play a role in both the mode
458 and amount of adaptation to similar selection pressures. Here we show that in a recent
459 evolutionary radiation, similar responses to environmental gradients and species history
460 were the largest predictors of trait variation, but that unique responses to selection were
461 also important in trait diversification. These varied responses suggest that even in a
462 closely related group, species are responding to similar selection pressures in different
463 ways and that evolutionary history can have an effect on both the size and direction of the
464 response to selection.

465 *1. Evolutionary history and similar responses to the environment play the largest roles in*
466 *trait diversification of the white proteas.*

467 The strongest predictor of multivariate trait variation is rainfall seasonality (Table
468 2), but the effect of history and other shared responses to the environment were nearly as
469 large. Unique responses to the environment, while detectable, played a smaller role in
470 trait differentiation. The large shared responses of traits to the environmental gradients
471 show that even though the species differ in their mean trait values (significant species
472 effect), many environmental responses are similar. This suggests that much trait

473 evolution within species represents parallel evolution to similar environmental gradients.
474 In particular, similar responses to rainfall seasonality (PPTCON) was the largest predictor
475 of multivariate trait variation. However, the significant unique responses to the
476 environment indicate that the species also respond differently to each environmental
477 gradient.

478 2. *The traits that drive the unique evolutionary response to the environmental gradients*

479 In spite of broad similarity in multivariate trait-environment associations detected
480 by the MANOVA, the six species of the white proteas differ in the degree to which their
481 traits are associated with the environment. Using the structural equation model we
482 detected 20 significant trait-environment associations in *P. mundii*, which might be due to
483 the large environmental differences between the eastern and western ranges of *P. mundii*.
484 In contrast, we did not detect any significant relationships between traits and the
485 environment in *P. venusta*. *P. venusta* is found only near the tops of mountains and does
486 not cover as much of the environmental gradients as do the other species. It also has
487 smaller population sizes than the other species (Rebelo 2006), so it might not harbor the
488 genetic variability necessary to respond to selection.

489 In most of the cases in which more than one species had a significant trait-
490 environment association, the differences were in the strength of the association, but in a
491 few cases there were differences in the direction of the association. In each of the 36 trait-
492 environment combinations (e.g. COLDPCA and seed mass) in which we found at least
493 one significant species-specific correlation, we found at least one species in which the
494 relationship was different from that of other species (either not significant, or in the
495 opposite direction). While others (Travisiano *et al.* 1995, Ruzzante 2003, Gomes &

496 Monteiro (2008), and Eroukhmanoff *et al.* 2009) have shown that evolutionary history
497 affects the magnitude of a selection response, very few studies show closely related
498 species responding to similar selection pressures in *opposite directions*.

499 All of the traits for which we detected trait-environment associations of opposite
500 signs were root traits, and in all cases, *P. aurea* varied in the opposite direction of *P.*
501 *mundii* or *P. punctata*. This result is especially surprising, because *P. aurea*'s
502 environmental ranges are quite similar to *P. mundii* (Figure 1) and its geographic
503 distribution lies between two disjunct population segments of *P. mundii*. It appears that *P.*
504 *aurea* and *P. mundii* populations are adapting to similar habitats in different ways. In two
505 out of the three cases when the two species have opposite trait-environment correlations,
506 trait variation in *P. mundii* reflects the pattern expected from broad-scale comparisons.
507 First, in areas with more concentrated rainfall, which in our dataset is strongly correlated
508 with total rainfall, *P. mundii* populations tend to have more branched root systems (lower
509 root length:mass ratio) in areas where rainfall is highly concentrated (higher total), while
510 *P. aurea* had more simple root systems. Nicotra *et al.* (2002) found that shrub species
511 from drier areas of Australia invested more in the main axis of their roots than those from
512 wetter areas, similar to the pattern that we detected in *P. mundii*. Second, *P. mundii* and
513 *P. punctata* individuals from warmer areas have heavier root systems (and shoots), but *P.*
514 *aurea* populations have lighter root systems (and non-significantly, shoots). Many studies
515 have shown that plants at higher elevations tend to be smaller than those at lower
516 elevations (Clausen *et al.* 1940, Woodward 1986, Oleksyn *et al.* 1998; Byars *et al.* 2007)
517 and in our system, winter temperature is strongly associated with elevation. In the third

518 case, we have no expectation for the association between root length:mass ratio and
519 temperature.

520 *3. Trait-environment associations rarely reflect large scale patterns.*

521 Some of the trait-environment associations that we detected were in the directions
522 that we expected based on trends documented in many other studies, but some were not,
523 and some expected relationships were not detected at all (Table 1). For example, large
524 seed size has long been considered an adaptation to low nutrient soils because of the
525 additional resources that can be stored in a larger seed (Beadle 1966, Stock *et al.* 1990,
526 Milberg *et al.* 1998, Vaughton & Ramsay 2001). Patterns consistent with this expectation
527 have been found in studies of two other members of the Proteaceae, *Banksia*
528 *cunninghamii* (Vaughton & Ramsey 2001) and *Protea compacta* (Shane *et al.* 2008). In
529 these studies, large seed size was shown to be an adaptive response to low nutrient soils.
530 In contrast to these expectations, we did not find any association between seed size and
531 soil fertility. *P. mundii* individuals from areas with more concentrated (and more) rainfall
532 did have larger seeds, but this might be a plastic response as a result of better maternal
533 environment.

534 In root traits, we expected to find a negative association between soil fertility and
535 the presence of proteoid roots, but we found no such association. Only in *P. subvestita*
536 did we find the expected relationship between intensity of dry-season drought and root
537 weight and root length:mass ratio. Seedlings from populations that experience stronger
538 droughts tended to have lighter, simpler roots systems (for a given length, they weighed
539 less). We found only one association between root:shoot biomass ratio and the intensity
540 of dry season drought, even though this association is a textbook example of a resource

541 investment trade-off (Larcher 1995) and has often been detected in other studies (e.g.
542 Blum 1996, Lloret *et al.* 1999, Li & Wang 2003). We detected this pattern in *P. mundii*,
543 which has a large range of drought intensities. Our failure to detect this association in
544 other species might reflect a smaller absolute range of drought intensities. Alternatively,
545 rooting depth and root:shoot ratios might have little to do with drought intensity in our
546 species. In *Protea*, rooting depth is often related to the depth of the water table (Richards
547 *et al.* 1995, Watt & Evans 1999), which depends on local geology. None of our
548 environmental variables captures variation in water table depth.

549 The relationships that we found between above-ground traits and the local
550 environment are consistent with those reported earlier for garden-grown plants by
551 Carlson *et al.* (2011). However, in most cases we only detected a significant association
552 in one or two species. For example, both studies found that plants from areas with more
553 concentrated precipitation have broader leaves (lower LWR), but in our study, it was only
554 *P. mundii* in which we could detect the association. Carlson *et al.* (2011) ascribe the
555 broader leaves in areas with more seasonal rainfall to the positive association between
556 PPTCON and total rainfall. We may be detecting a relationship between leaf shape and
557 total rainfall, a pattern seen across the Proteaceae (Thuiller *et al.* 2004, Yates *et al.* 2010).
558 Narrow leaves lose heat through convection more efficiently than do wider leaves (Yates
559 *et al.* 2010) and thus are likely to be favored in drier areas where transpirational cooling
560 is particularly costly. Carlson *et al.* (2011) found that the SLA of plant leaves grown in
561 two common gardens was associated with differences in COLDPCA and DRYPCA of
562 their source populations. In our study, only *P. aurea* populations varied in SLA along the
563 temperature gradient (COLDPCA) and no species had a significant association between

564 SLA and drought. Our failure to find strong associations between SLA and the
565 environmental gradients in the greenhouse might be due to G X E interactions. The mild
566 conditions in the greenhouse might have resulted in smaller differences in leaf
567 morphology between populations (values of all traits for all populations are included in
568 Supplemental Table 3). Alternately, the lack of significant associations that we detected
569 in the greenhouse might be due to differences in power between the two analysis. In
570 Carlson *et al.* (2011) the trait-environment associations were estimated using all of the
571 populations in all of the species, whereas in the study presented here, there were only a
572 maximum of 5 populations per species to be used to detect an association between traits
573 and the environment.

574 **Conclusions**

575 Our study shows that even in a closely related group of species, evolutionary
576 history can play a role in trait diversification. While on average, the species responded
577 similarly to the environment, they also had unique responses. In particular, the structural
578 equation model revealed that in some cases, the root traits of *P. aurea* and *P. mundii*
579 varied in opposite directions in response to the same environmental gradient.
580 Evolutionary history can have an effect on adaptation at large and small evolutionary
581 scales. Here we document how evolutionary history can affect trait-environment
582 associations even between very closely related species. We show that evolutionary
583 outcome of selective pressures cannot always be predicted. Further – the lack of
584 correspondence between large scale trait-environment associations and those that we
585 detect within the white proteas suggests that these gradients are unlikely to be driving
586 much of the trait diversification in this group.

587

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598

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783 Table 1: Expected trait-environment association, the corresponding trait and environment used to detect the association in this study,
 784 and the species and direction in which the pattern was found. + indicates that the trait-environment association was in the expected
 785 direction, - indicates that the trait-environment association was in the opposite direction from the expectation.

Expected Trait-environment				
Correlation	Citation	Trait	Environment	Supported in our study?
Biomass decreases as elevation increases	Clausen <i>et al.</i> 1940	root mass, shoot mass, shoot height, leaves present	COLDPCA	+ <i>P. mundii</i> , + <i>P. punctata</i> , + <i>P. laticolor</i> , - <i>P. aurea</i>
Root:shoot ratio increases with decreased rainfall	Schenk & Jackson 2002	root mass/shoot mass	DRYPCA	+ <i>P. mundii</i>
Leaves become larger and wider with increased rainfall	Cunningham <i>et al.</i> 1999	LWR	DRYPCA	+ <i>P. aurea</i> and + <i>P. mundii</i>
Plant height increases with increased rainfall	Moles <i>et al.</i> 2009	shoot height	DRYPCA	No
Roots length increases with increased drought	Canadell <i>et al.</i> 1996	root length	DRYPCA	No
SLA increases with increased	Wright <i>et al.</i>	SLA	DRYPCA	No

rainfall	2004			
Seeds are larger in lower nutrient soils	Beadle 1966	seed mass	FERTPCA	No
Proteoid roots increase with decreased soil fertility	Lamont 1982	proteoid roots	FERTPCA	No
SLA decreases with decreased soil fertility	Cunningham <i>et al.</i> 1999	SLA	FERTPCA	No
Root branching increases with more concentrated rainfall	Nicotra <i>et al.</i> 2002	root length / root mass	PPTCON	+ <i>P. mundii</i> , - <i>P. aurea</i>
Root depth increases with increasing rainfall seasonality	Schenk & Jackson 2002	root length	PPTCON	+ <i>P. subvestita</i>

786 Table 2: MANOVA table including Wilks's η^2 , the partial variance explained by each factor, and 95% confidence intervals around the
 787 Wilks's η^2 , estimated by jackknifing.

test for	factor	Wilks's λ	Approx. F	df	Wilks's η^2	p	2.5% CI	97.5% CI
shared response to environment	COLDPCA	0.812	4.466	14, 270	0.188	<0.0001	0.183	0.195
	DRYPCA	0.783	5.346	14, 270	0.217	<0.0001	0.211	0.222
	FERTPCA	0.749	6.454	14, 270	0.251	<0.0001	0.245	0.257
	PPTCON	0.591	13.362	14, 270	0.409	<0.0001	0.403	0.416
history	species	0.132	9.746	70, 1289.545	0.333	<0.0001	0.331	0.335
unique response to environment	COLDPCA \times species	0.471	2.92	70, 1194.326	0.140	<0.0001	0.137	0.143
	DRYPCA \times species	0.612	1.856	70, 1194.326	0.094	<0.0001	0.092	0.096
	FERTPCA \times species	0.673	1.479	70, 1194.326	0.076	<0.01	0.074	0.079
	PPTCON \times species	0.622	1.786	70, 1194.326	0.09	<0.001	0.089	0.093

788 Table 3: Summary statistics of the relationships estimated in the Structural Equation
 789 Model; mean, standard deviation, and 95% credible intervals.

	mean (β)	sd	2.50%	97.50%
Trait-Latent Variable relationships				
SLA-Leaf	-0.316*	0.073	-0.464	-0.176
Leaf LWR-Leaf	-0.231*	0.073	-0.376	-0.09
Leaf Mass-Leaf	1.054*	0.037	0.984	1.13
Root dead-Root	-2.962*	0.433	-3.874	-2.183
Root length:mass-Root	0.191*	0.044	0.106	0.276
Root weight-Root	0.497*	0.032	0.436	0.559
Proteoid Roots-Root	1.402*	0.394	0.67	2.188
Root: Shoot Mass-RootShoot	0.514*	0.053	0.411	0.617
Shoot Height-Shoot	1.245*	0.114	1.032	1.477
Shoot Weight-Shoot	1.8*	0.111	1.592	2.027
External - Latent Variable relationships				
Daystogerm - Leaf	-0.035	0.038	-0.113	0.039
Daystogerm - Root	-0.11*	0.056	-0.223	-0.002
Daystogerm - RootShoot	-0.016	0.037	-0.087	0.056
Daystogerm - Shoot	-0.023	0.016	-0.055	0.009
Fertilizer - Leaf	0.093	0.077	-0.061	0.241
Fertilizer - Root	-0.277*	0.11	-0.496	-0.056
Fertilizer - RootShoot	-0.046	0.088	-0.216	0.131
Fertilizer - Shoot	0.031	0.045	-0.059	0.121

Seed weight - Leaf	0.384*	0.077	0.231	0.533
Seed weight - Root	0.218*	0.106	0.013	0.42
Seed weight - RootShoot	0.005	0.082	-0.152	0.172
Seed weight - Shoot	0.227*	0.035	0.161	0.297
Seed weight - Daystogerm	-0.188	0.108	-0.404	0.023
Relationships between Latent Variables				
Root - Shoot	0.557*	0.047	0.46	0.645
Leaf - Root	0.602*	0.04	0.519	0.679
Leaf - Shoot	0.843*	0.022	0.795	0.88
Shoot - RootShoot	-1.473*	0.18	-1.844	-1.128
Root - RootShoot	1.104*	0.046	1.018	1.198

790 Note: The relationships between traits and latent variables are one way, but the
791 relationships among Root, Shoot, and Leaf latent variables are reciprocal. The
792 relationships between the RootShoot latent variable and Root and Shoot latent variables
793 are also one-way with Root and Shoot informing RootShoot. * indicates relationships for
794 which the 95% credible intervals do not overlap zero.
795

796 Figure legends:

797 Figure 1: White protea climate envelopes in three dimensions: COLDPCA (PCA
798 summarizing coldness of winter), DRYPCA (PCA summarizing intensity of summer
799 drought), and PPTCON (rainfall seasonality). Values were obtained by intersecting the
800 locations of all observed populations of each species from the Protea Atlas (a database of
801 250k presence/absence observations of all species in the Proteaceae; Rebelo 2006) with
802 the South African Atlas of Agrohydrology (Schulze 2007).

803

804 Figure 2: Sampling locations and ranges of the white protea species. Inset is a map of
805 Africa, with enlarged area in the box.

806

807 Figure 3: Relationships between latent variables, measured traits, and outside factors
808 estimated in the SEM analysis. Latent variables are represented by grey ovals and traits
809 and outside factors (seedwt=seed weight, daystogerm=# of days to germination,
810 fert=fertilizer treatment) are represented by white boxes. All variables are standardized,
811 and the relative size of each relationship is indicated by the width of the arrow that
812 connects the variables. Black arrows indicate positive relationships and grey arrows
813 indicate negative ones. Dotted arrows are non-significant relationships. Asterisks indicate
814 the traits for which the regression coefficients were set to 1.

815

816 Figure 4: Correlations between measured traits and the local environment. The
817 environment is summarized along four axes COLDPCA (coldness of winter), DRYPCA
818 (intensity of summer drought), and PPTCON (rainfall seasonality). All traits and

819 environments are standardized and the size of each circle is proportional to the size of the
820 effect. Black circles are positive relationships, grey circles are negative. Only significant
821 relationships are shown. Black arrows indicate reversals in trait-environment correlations.
822 *P. venusta* is excluded from the figure because it had no significant trait-environment
823 correlations.